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14. ABSTRACT
This predoctoral training grant, supported by the Department of Defense Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP), aims to study the possible role of ABC transporters in altered apoptotic signal transduction in drug sensitive and resistant cells, by a combination of molecular biological, biochemical, and biophysical methods. The first year of the funding period was spent learning basic molecular biology techniques while working with a model system. The plasmodial homologue of human Pgp, PfMDR1, was heterologously expressed in yeast and its function in response to drugs measured in terms of ATPase activity. The data from this project have already led to a quality journal publication (with more forthcoming), and the knowledge and skills I have gained will undoubtedly be extremely valuable in future endeavors with human Pgp. Preparations in terms of mammalian cell culture and microscopy methodology have been made for the start of actual Pgp research, and the timetable as outlined in the original grant proposal is still intact.

15. SUBJECT TERMS
Drug resistance, ABC transporters, model systems, heterologous expression

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INTRODUCTION

Members of the ATP-binding cassette (ABC) superfamily are proteins composed of transmembrane domains (TMDs) and nucleotide binding domains (NBDs), usually organized in single or tandem cassettes of 6 TM helices followed by a single NBD. It is well established that one of the human members of this family, P-glycoprotein (Pgp), is involved in resistance to chemotherapeutic agents. Pgp is a 170 kDa plasma membrane glycoprotein encoded by the *mdr1* gene (Juliano & Ling 1976, Roninson *et al.* 1986) that is currently postulated to act as a drug efflux pump that decreases the intracellular concentration of chemotherapeutic agents (Dano 1973). However, many studies also indicate that Pgp is likely to have additional functions linked (in a general way) to signal transduction and ion transport. This predoctoral training grant, supported by the Department of Defense Breast Cancer Research Program (BCRP) of the Office of the Congressionally Directed Medical Research Programs (CDMRP), aims to study the possible role of ABC transporters in altered apoptotic signal transduction in drug sensitive and resistant cells, by a combination of molecular biological, biochemical, and biophysical methods.

BODY

I have spent most of my first year learning basic molecular biology techniques while working with a model system. The genome of *Plasmodium falciparum*, the protozoan parasite responsible for causing malaria, codes for an ABC protein with high homology to human Pgp. This protein, known as *P. falciparum* multidrug resistance protein 1 (*Pf*MDR1) or P-glycoprotein homologue 1 (Pgh1), has been postulated to influence parasite drug resistance to a variety of antimalarial compounds. Like Pgp, *Pf*MDR1 is composed of two ABC cassettes, each with 6 TM helices followed by an NBD. The proposed substrates for each protein also share some chemical characteristics (Figure 1), and the resistance conferred by both proteins is reversible by the calcium channel blocker verapamil. Thus, PfMDR1 function vs. these drugs would be a useful comparison in elucidating aspects of Pgp function.

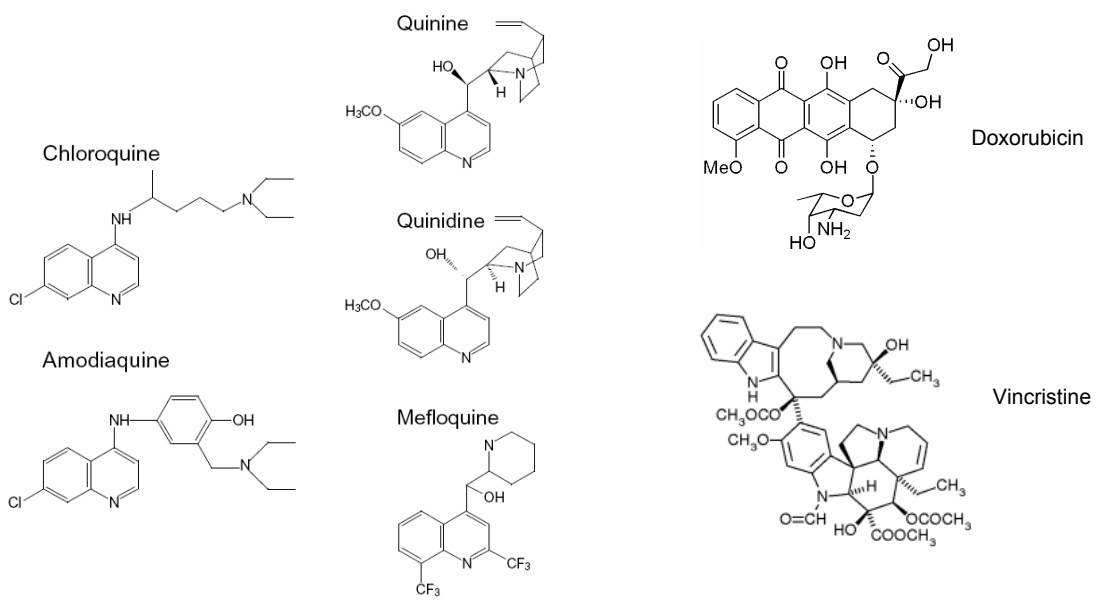


Figure 1. Chemical structures of antimalarial (*left*) and chemotherapeutic (*right*) drugs. Each of the proposed Pgp and PfMDR1 substrates is a polyaromatic, amphipathic weak base with a titratable nitrogen with a pKa between 6 and 9.

However, due to the complexities of the parasite-host interaction, this goal would benefit from the heterologous expression of the protein in a simpler system. Due to the unusually high AT content of the malarial genome, the *pfmdr1* gene (75% AT) cannot be efficiently heterologously expressed as native cDNA. Therefore, we backtranslated the protein into a nucleotide sequence codon-optimized for expression in *Pichia pastoris* yeast. Yeast were transformed with a construct encoding the N-terminal cassette, the C-terminal cassette, or full length *Pf*MDR1 (fused to a polyhistidine tag and a biotin acceptor domain) under control of the alcohol oxidase promoter (Figure 2). Upon methanol induction, the yeast expressed high levels of the malarial protein (Figure 3).

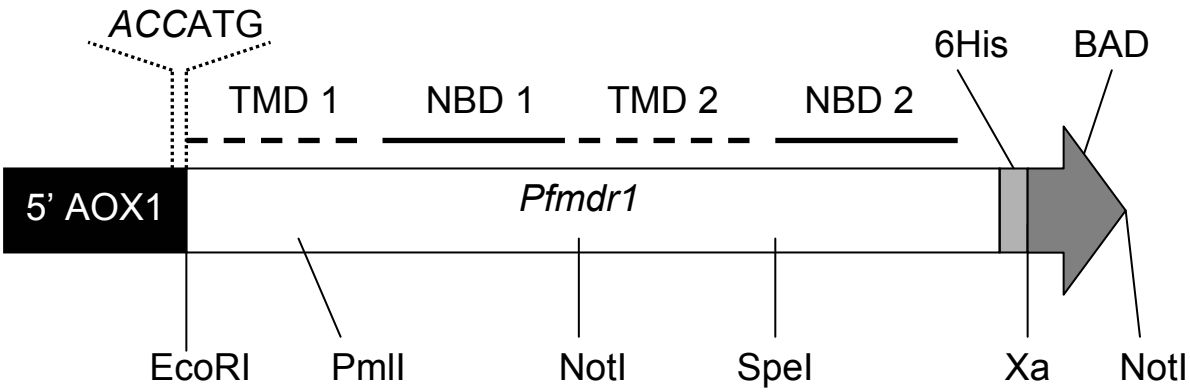
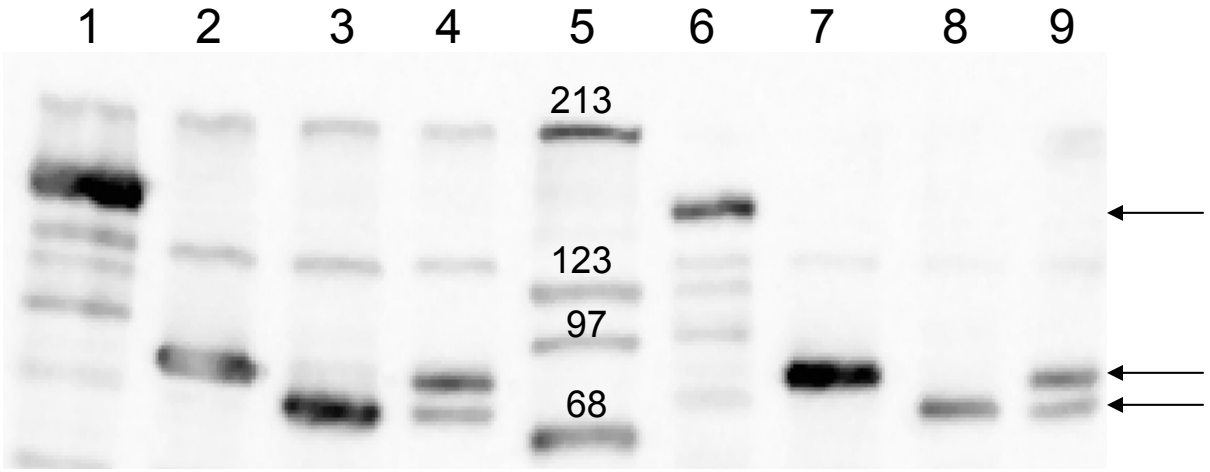


Figure 2. Schematic of the "yeast optimized" PfMDR1 gene created for this work. The position of the engineered Kozak sequence, restriction sites unique to the ORF, predicted TMDs and NBDs, as well as fused hexa histidine and BAD are indicated.



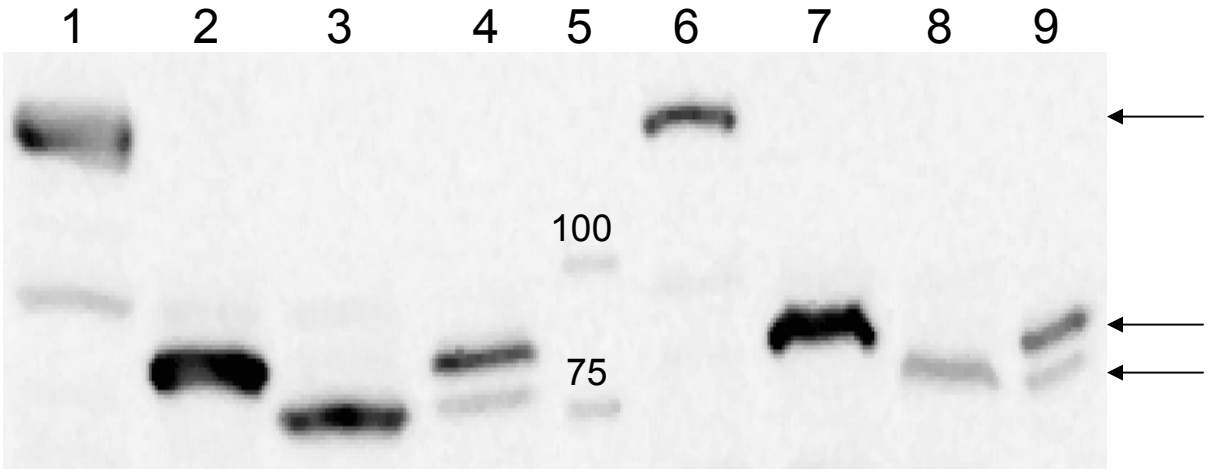


Figure 3. Parallel biotin (*top*) and polyhis (*bottom*) immunoblots of wildtype *Pf*MDR1 constructs. Full length PfMDR1 migrates at 161.69 kDa (top arrow on right; predicted mass = 171.81 kDa), N-terminal polypeptide migrates at 87.44 kDa (middle arrow; predicted mass = 94.69 kDa), and the C-terminal migrates at 77.03 kDa (lower arrow; predicted mass = 86.94 kDa). Lanes 1 – 4 are crude yeast membrane fractions, lane 5 is molecular weight standards, lanes 6 – 9 are purified plasma membrane fractions. Lanes 1, 6 contain full length PfMDR1; lanes 2, 7 contain N-terminal half transporter; lanes 3, 8 contain C-terminal half transporter; lanes 4, 9 show both N and C half transporters co-expressed in the same yeast.

While it is currently controversial as to whether it is overexpression or mutation of P*f*MDR1 that results in drug resistance (Price *et al* 2004, Reed *et al* 2000), we utilized site-directed mutagenesis to create *Pf*MDR1 isoforms harbored by several commonly used lab strains (Table 1).

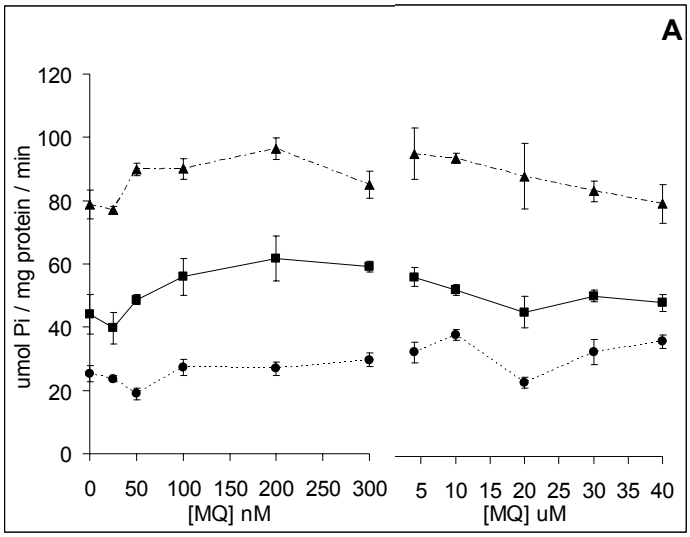
Allele	N86Y	Y184F	S1034C	N1042D	D1246Y
3D7, D10 (wildtype)	N	Y	S	N	D
Dd2	Y	Y	S	N	D
7G8	N	F	C	D	Y

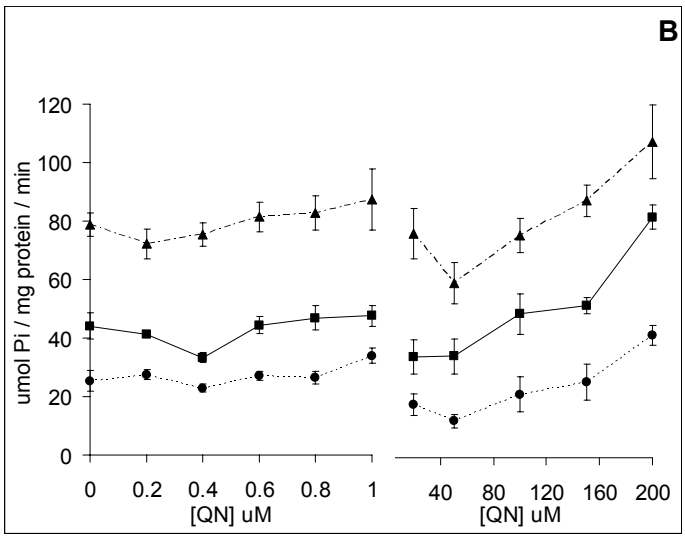
Table 1. Mutations in *Pf*MDR1 associated with drug resistance. 3D7 (derived from an isolate taken from a passenger at a Netherlands airport) and D10 (from Papua New Guinea) are chloroquine resistant strains, whereas Dd2 (Thailand) and 7G8 (Brazil) are chloroquine resistant. In addition, Dd2 resistance is verapamil-reversible but 7G8 is not.

Since ABC proteins by definition bind and hydrolyze ATP, an obvious test of their function is ATPase activity. Our lab has previously used a colorimetric assay performed in borosilicate glass tubes to detect the release of orthophosphate, but this method was laborious and prone to high error. Therefore, we invested a significant amount of time and effort into reconfiguring the assay on a ¼ scale so as to be performed in 96-well plates amenable to the use of multichannel pipettes. In this manner, we are able to simultaneously test several samples under a myriad of drug combinations with multiple replicates. We then compared the activity of purified yeast plasma membranes harboring various *Pf*MDR1 isoforms under basal conditions (Table 2) as well as in the presence of antimalarial drugs (Figure 4).

	3D7	Dd2	7G8
pH optimum	7.0	7.5	7.0
V_{max} (μmol Pi / mg / min)	62.9	109.9	42.7
K_m (mM)	2.14	2.00	3.42
Vanadate IC_{50} (μM)	2.25	4.00	1.25
Concanamycin IC_{50} (nM)	82.6	67.4	42.9

Table 2. Kinetic parameters and inhibitor sensitivities of *Pf*MDR1 isoforms. All isoforms of the protein have relatively high K_m and V_{max} for ATP hydrolysis, but similar vanadate and concanamycin sensitivities, compared to other known ABC proteins.





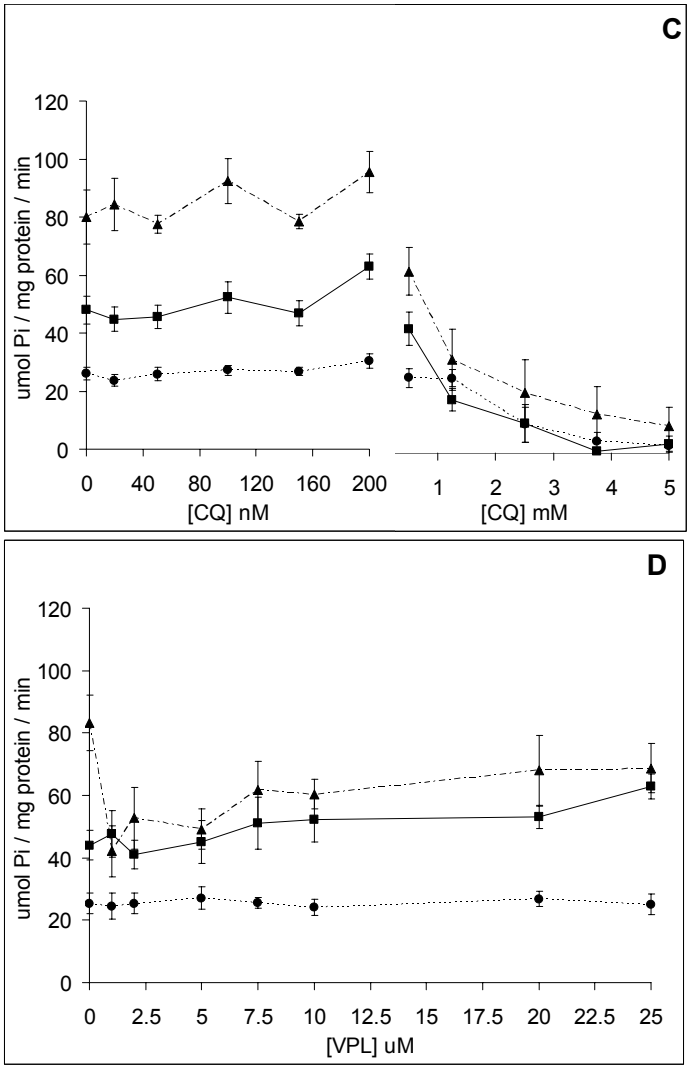


Figure 4. Effect of antimalarial drugs on *Pf*MDR1 ATPase activity: mefloquine (A), quinine (B), chloroquine (C), and verapamil (D) profiles of wildtype (squares, solid line), Dd2 (triangles, dashed line), and 7G8 (circles, dotted line) *Pf*MDR1. The left side of graphs A-C corresponds to drug concentrations found in the cytoplasm of malarial parasites while the right reflects to the environment of the parasite digestive vacuole.

Drug resistance-associated *Pf*MDR1 mutants show either elevated (Dd2) or reduced (7G8) basal ATPase activity and different patterns of drug stimulation or inhibition, relative to wildtype. Reports of how Pgp ATPase activity is altered by the drugs to which it confers resistance vary (Loo & Clarke 1995, Sharom *et al* 1995, Regev *et al* 1999), but with a few exceptions they are stimulatory and typically at least 2 – 3 fold, occasionally even as high as 10 – 20 fold. In contrast, *Pf*MDR1 is only mildly stimulated by its proposed substrates. The largest drug effect obtained was a strong inhibition by high concentrations of chloroquine (Figure 4C), a drug to which *Pf*MDR1 is not believed to confer resistance. Additionally, the only substantial effect of verapamil alone was inhibition of the Dd2 isoform at 1 μM (Figure 4D).

Since Dd2 has only one mutation relative to wildtype, any differences in ATPase activity may be ascribed to N86Y. However, so it is more difficult to partition responsibility amongst the four mutations present in 7G8. In order to elucidate the mechanism behind the conspicuously

low basal activity of 7G8, we created a series of strains harboring various combinations of mutations. The single S1034C mutation as well as the full C-terminal S1034C/N1042D/D1247Y mutant both recapitulate the 7G8 level of activity (Figure 5).

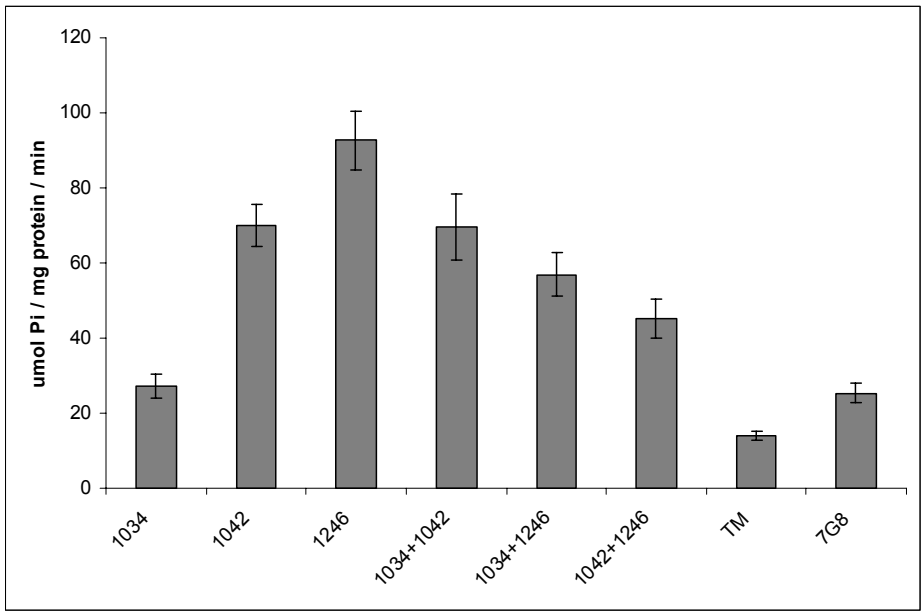


Figure 5. Basal ATPase activity of partial-7G8 mutants.

We are currently preparing a short communication manuscript outlining this and other discoveries about the 7G8 isoform, including an analysis of comparisons to similar studies of Pgp. For example, the 1034 mutation is located within TM helix 11, which has been shown by photoaffinity labeling to comprise part of the drug binding site in Pgp (Greenberger 1993, Morris *et al* 1994).

KEY ACCOMPLISHMENTS

Research Accomplishments

- Mastery of Roepe lab protocols including codon-optimized gene design, nested PCR gene assembly, bacterial transformation, side-directed mutagenesis, yeast transformation, yeast culture, crude membrane and plasma membrane preparation, affinity column chromatography, and ATPase assay
- Creation of yeast strains overexpressing wildtype and various mutant *Pf*MDR1 isoforms
- Development and optimization of a high-throughput 96-well plate-based ATPase assay
- Identification of key mutations responsible for increased and decreased protein activity in ABC transporters

Training Accomplishments

- Successful completion of coursework and the Tumor Biology Comprehensive Examination

- Attendance of numerous seminars concerning breast cancer presented by the Lombardi Comprehensive Cancer Center and the Georgetown University Department of Oncology
- Participation in weekly seminars and journal clubs addressing both cancer-specific and basic science research, intended to stimulate awareness of other areas of research and new laboratory techniques that may inspire my own research

REPORTABLE OUTCOMES

- Article in press: Amoah LE, Lekostaj JK and Roepe PD. (2007) Heterologous expression and ATPase activity of mutant vs. wildtype PfMDR1 protein. *Biochemistry*.
- Abstract/poster: "Heterologous expression, purification, and ATPase activity of PfMDR1 protein" Woods Hole Molecular Parasitology Meeting, September 10-14, 2006
- Abstract/poster: Georgetown University Center for Infectious Disease Annual Conference, February 26, 2007
- Presentation: "Drug resistance mediated by mdr proteins" Georgetown University Tumor Biology Graduate Student Data Meeting, July 20, 2006

CONCLUSION

Over the past year, I have begun my training as a scientist in the field of drug resistance. I have received extensive training in basic molecular biology and I have gained experience working with an ABC homologue in a model system. The techniques that I have learned working on this project will undoubtedly be extremely valuable when working with human Pgp. I have begun learning fluorescence microscopy and single cell photometry (SCP), including the use of the Roepe lab's custom perfusion chamber and appropriate software for data analysis. I have also recently obtained MCF7 breast cancer cell lines (parental and Pgp-transfected) from the laboratory of Robert Clarke in the Lombardi Cancer Center and I am beginning to optimize growth culture conditions for use in SCP experiments.

During the second year of the grant, I will more directly address drug resistance phenomena in the realm of breast cancer. I hope to complete Task One as outlined in the statement of work of my original grant proposal: "To measure perturbations in plasma membrane ion transport for drug sensitive vs. resistant breast cancer cells by live cellular imaging methods (months 1 – 18)."

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APPENDICES - None